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EUROPEAN PATENT APPLICATION

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Process for preparing a macrolide.

(c) A process for preparing tylactone (20-dihydro-20,23-dideoxytylonolide), which has the formula:

CH3 CH3 CH3 CH3 CH3

by submerged serobic fermentation of Streptomyces tradise NRRL 12188 or a tylactone-producing mutant or recombinant

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PROCESS FOR PREPARING A MACROLIDE

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preparation of the macrolide 20-dihydro-20,23-dideoxy-This invention relates to a process for the

venience hereinafter. Tylactone has the structure 1: tylonolide, which will be called tylactone for con-

wherein R and R_L = an acyl moiety.

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hours until esterification is substantially complete. about room temperature for from about 1 to about 24 organic solvent (for example, pyridine) at about 0°C to acylating agent, such as an acyl anhydride, in an stoichiometric quantity (or a slight excess) of an as, for example, treatment of the compound with a fication techniques generally known in the art, such The derivatives can be prepared by esteri-

purified by known techniques. Once formed, the acyl derivatives can be separated and those described by Okamoto et al. in U.S. 4,092,473.

carried out enzymatically using procedures such as dicyclohexylcarbodiimide. Acylations can also be organic acid and a dehydrating agent such as N,N'-Acylation can also be achieved by using a mixture of an acid scavenger) and active esters of organic acids. halides (usually in combination with a base or other

Typical acylating agents include anhydrides,

macrolide antibiotics.

useful as intermediates in the preparation of new The acyl ester derivatives of tylactone are treatment with acylating agents using methods known in 5-hydroxyl groups to give acyl ester derivatives by

Tylactone can be esterified at the 3- and

tical to that of tylosin. herein, the stereochemistry of the compounds is idenical assignments are indicated in the structures given antibiotics can be prepared. Although no stereochemuseful intermediates from which 16-membered macrolide

The compounds of structures 1 and 2 are

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preferred. Thus, for example, preferred carbon sources 30 product isolation, however, certain culture media are economy in production, optimal yield, and ease of myces fradiae can be any one of a number of media. For The culture medium used to grow the Streptodesired compound is produced. 52 able culture medium until a substantial amount of the compound under submerged aerobic conditions in a suitstrain of Streptomyces fradiae which produces this Tylactone can be prepared by culturing a maleic, fumaric, malonic and phthalic acids. 50 esters derived from dicarboxylic acids such as succinic, aromatic moiety. Suitable esters also include hemihalogen, nitro, lower alkoxy and the like on the aralkyl- acids optionally bearing substituents such as aryl-, and aralkyl-sulfonic acids, the aryl- and SI acetic, mandelic and 2-thienylacetic acids, and alkyl-, adamantanecarboxylic, benzoic, phenylacetic, phenoxycyclohexanecarboxylic, β-cyclohexylpropionic, lalkoxycarbonic, stearic, cyclopropanecarboxylic, acetic, propionic, butyric, isovaleric, glucuronic, OT derived from acids such as formic, acetic, chloro- . Representative suitable esters include those scids, such as sulfuric and phosphoric acids sadding a particular solids. scids of from 1 to 18 carbon atoms, and of inorganic pererocyclic carboxylic, sulfonic and alkoxycarbonic and including aliphatic, cycloaliphatic, aryl, aralkyl, Useful esters are those of organic acids chromatography and crystallization. mixture by standard procedures such as extraction, The ester derivative can be isolated from the reaction

fragments of the organism to obtain a fresh, actively volume of culture medium with the spore form or mycelial tative inoculum is prepared by inoculating a small is preferable to use a vegetative inoculum. The vege-52 of large tanks with the spore form of the organism, it lag in production commonly associated with inoculation obtained by shake-flask culture. Because of the time preferred. Small quantities of tylactone may be $t_{\rm Y}$ lactone submerged aerobic fermentation in tanks is 02 For production of substantial quantities of problem. large-scale fermentation media if foaming becomes a such as polypropylene glycol (M.W. about 2000) to add small amounts (i.e. 0.2 ml/L) of an antifoam agent SI requirements of the organism. It may be necessary to the medium in amounts sufficient to meet the growth commonly occur as impurities in other constituents of included in the culture medium. Such trace elements growth and development of the organism should also be OT Essential trace elements necessary for the chloride, carbonate, sulfate, nitrate, and like ions. potassium, sodium, magnesium, calcium, ammonium, customary soluble salts capable of yielding iron, can be incorporated in the culture media are the ς and the like. Among the nutrient inorganic salts which include corn meal, soybean meal, fish meal, amino acids such as soybean oil. Preferred nitrogen sources as dextrin, glucose, starch, and corn meal and oils in large-scale fermentation include carbohydrates such

inoculum is then transferred to a larger tank. The

growing culture of the organism. The vegetative

can also be used. as that used for larger fermentations, but other media medium used for the vegetative inoculum can be the same

The method of this invention comprises

as a major component. only minimal amounts of tylosin, but produces tylactone which produces tylosin. The new microorganism produces chemical mutagenesis of a Streptomyces fradiae strain culturing a new microorganism which was obtained by OT

Research, North Central Region, 1815 North University of the Northern Regional Research Center, Agricultural A culture of this microorganism has been organism is also classified as a strain of Streptomyces organism which produces tylactone. The new micro-This invention also relates to the new micro-

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Street, Peoria, Illinois, 61604, from which it is

12188. available to the public under the accession number NRRL deposited and made part of the stock culture collection 20

of this invention. the characteristic of tylactone production are a part binants of Streptomyces fradiae NRRL 12188 which retain All natural and induced variants, mutants and recomgamma rays, and N-methyl-N'-nitro-N-nitrosoguanidine. chemical mutagens, such as ultraviolet light, X-rays, obtained by treatment with various known physical and tants or variants of the NRRL 12188 strain may be subject to variation. For example, recombinants, mucharacteristics of Streptomyces fradiae NRRL 12188 are As is the case with other organisms, the

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(generally without pH adjustment) with a suitable the filtered broth involves extracting the broth processes. A preferred technique for purification of variety of techniques may be used in the extraction both the filtered broth and the mycelial cake. 52 tration of the fermentation broth and extraction of 1) extraction of the fermentation broth or 2) filconery of tylactone, therefore, can be accomplished by soluble in the medium in which it is produced. bility of tylactone in water, it may not be altogether 20 in the fermentation art. Because of the limited solurecovered from the fermentation medium by methods used aerobic fermentation conditions, tylactone can be Following its production under submerged ·[(8791) ST Kennedy in J. Chromatographic Science, 16, 492-495 with a UV detection system [see, for example, J.H. broth, using high-performance liquid chromatography during the fermentation by testing samples of the Production of tylactone can be followed OT pressure). about 30% or above (at 28°C and one atmosphere of cent of air saturation for tank production should be For efficient antibiotic production the perprocesses, sterile air is bubbled through the culture ς As is customary in aerobic submerged culture atures of about 28°C. production of tylactone appears to occur at temperperatures between about 10° and about 40°C. Optimum S. fradiae NRRL 12188 can be grown at tem-

solvent such as amyl acetate or petroleum ether, con-

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metabolic studies. The moteties, the metabolic portion or the added sugar moteties, the metabolic	30
By labeling erries are classes.	
io the preparation of labeled compounds for	
whe compound of structure I areaden	
which may be used to obtain the selected strains is	52
commenced to contract to contr	4
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animateh of grantine if they produce transfer	
to small shake-flask curtures of enotation	20
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except that it is blocked in tylactone formation can be	
blocked in ryids of producing tylosin A strain which is capable of producing tylosin	
• notability of the formation of the for	
is capable of producing tylosin except can	٥٦
The same and the same of the s	
(I) can be broconverting microorganism. The growing culture of a bioconverting microorganism.	
(1) can be bioconverted to tylosin by adding it to a	
antibiotics can be prepared.	
seful intermediates from annous example, tylactone	, 5
The compounds to macrolide assetul intermediates from which l6-membered macrolide	
The compounds of structures 1 and 2 are	£
rystals or an orrest chromatography.	၁
entrating the organic form oil is obtained, it may be rystals or an oil. If an oil is obtained, it may be	o '.
entrating the organic phase under vacuum to give	• 30

pathway of tylosin can be ascertained.

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In order to illustrate more fully the operation of this invention, the following examples are

Shake-flask Fermentation of Tylactone

A lyophilized pellet of Streptomyces fradiae.

NRRL 12188 was dispersed in 1-2 ml of sterilized water.

A portion of this solution (0.5 ml) was used to inoculate a vegetative medium (150 ml) having the following composition:

Composition:

Lingredient

Corn steep liquor

Yeast extract

1.0

Yeast extract

0.5

1.0

Yeast extract

0.5

Soybean oil (crude)

Deionized water

Alternatively, a vegetative culture of S.

fradise NRRL 12188 preserved, in 1-ml volumes, in

liquid nitrogen was rapidly thawed and used to inoculated the vegetative medium. The inoculated vegetative medium was incubated in a 500-ml Erlenmeyer flask at medium was incubated in a 500-ml Erlenmeyer flask at medium was incubated in a 500-ml Erlenmeyer flask at apout 300 rpm.

This incubated vegetative medium (0.5 ml) was used to inoculate 7 ml of a production medium having the following composition:

provided:

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•noijulos	ИЗОН	% 05	мұғр	2. 8	¢0	adjusted	MSS	Hq	Тре	30
40,444								ょ	Mate	
		1.76				īnge)	:D) U	ŗųa	Leci	
	_	2.0 0.0				(cznge)	lio ,	ези 3	Soyb Soyb	
		€.0				n:	x£zsc			52
		2. 0							Soyb	
		5.0				, rdnor				
	(8)	J.0	ошА		•.	que	regi	buI		

In order to provide a larger volume of inocular, 60 ml of incubated vegetative medium, prepared in section A, was used to inoculate 38 L of a second-stage vegetative your drowing composition:

closed-box shaker at 300 rpm.

B. Tank Fermentation of Tylactone

The inoculated fermentation medium was incubated in a 50-ml bottle at 29°C. for about 6 days on a

	Deroursed water	01
95.16	Soybean oil (crude)	
0.5	ε ^{ΟΌ} ΑΟ	
2.0	(NH ₄) 2 ^{HPO} 4	
*0.0	NaCL	
τ.0	Corn gluten	S
. 6.0	Land daiq	
6.0	Corn meal	
g°T	Beet molasses	
0.2		
(a) amound	Ingredient	

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(%) JRWOMA

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The inoculated production medium was allowed to ferment in a 68-liter tank for about 5 days at a temperature of 28°C. The fermentation medium was serated with sterile air to keep the dissolved oxygen level between about 30% and 50% and was stirred with conventional agitators at about 300 rpm.	25
The pH was adjusted to 7.2 with 50% NaOH solution.	
Soybean oil (crude) 3.15 0.09 Lecithin 90.90	ST
(NH _d) 2,10	
СаСО ₃ 0.10	
Corn gluten	στ
Fish meal 1.57 Corn meal	
Ingredient Amount (%)	

(%) JunomA duction medium having the following composition: pared was used to inoculate 40 liters of sterile pro-Incubated second-stage medium (4 L) thus pre-

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incubated in a 68-liter tank for about 47 hours at This second-stage vegetative medium was

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percentage elemental composition: carbon, 70%; hydro-	30
about 162-163°C. It has the following approximate	
from hexane or ethyl acetate-hexane and which melts at	
Tylactone is a white solid which crystallizes	
in chloroform is presented in the accompanying drawing.	
The infrared absorption spectrum of tylactone	52
T62-163°C.	
benzene-hexane or hot hexane to give about 2 g, m.p.	
orated under vacuum. Tylactone was crystallized from	
Fractions containing tylactone were combined and evap-	
acetate (9:1) to separate and isolate tylactone.	20
remove lipid substances, then with benzene:ethyl	
detection. The column was first eluted with benzene to	
(3:2) solvent system and conc. sulfuric acid spray for	
layer chromatography, using a benzene:ethyl acetate	
benzene. Elution is monitored by silica-gel thin-	ST
grade 62, Davison Chemical Co.) column, packed with	
chromatographed over a 5.25 x 36 in. silica-gel (Grace,	
dissolved in benzene (5 L). The benzene solution was	
concentrated under vacuum to give an oil. The oil was	
reading at 282 nm but no antimicrobial activity) was	στ
acetate extract (which has a high optical density	
was extracted with amyl acetate (400 L). The amyl	
by the addition of 2% sodium hydroxide. The filtrate	
Corp.). The pH of the filtrate was adjusted to about 9	
(3% Hyflo Supercel, a diatomaceous earth, Johns Manville	S
described in Example 1, was filtered using a filter aid	s •
Fermentation broth (1600 L), obtained as	
Isolation of Tylactone	٠.
Example 2	
	_

by silica-gel thin-layer chromatography. Sulfuric acid Tylactone can be distinguished from tylosin .abixolluz diethyl ether, petroleum ether, benzene and dimethyl methanol, ethanol, dimethylformamide, chloroform, 52 but is soluble in organic solvents such as acetone, Tylactone is nearly insoluble in water, aqueous dimethylformamide indicates it has no titrata-Electrometric titration of tylactone in 66% 20 [α]_{Σ2} -22.23° (<u>c</u> 1, ^{CH}3^{OH}). Tylactone has the following specific rotation: $E_{LC} = E_{LC} = E$ tylactone in neutral ethanol exhibits an absorption ST The ultraviolet absorption (UV) spectrum of 840 (medium), 820 (very small) and 661 (small). , (medium), 911 (shoulder), 859 (small), 868 (medium), small), 1025 (medium), 984 (very strong), 958 (strong), (strong), 1103 (medium), 1078 (medium), 1049 (very JO (strong), 1284 (medium), 1181 (very strong), 1143 1441 (sponider), 1404 (strong), 1379 (small), 1316 strong), 1626 (small), 1592 (very strong), 1458 (strong), (weak), 2353 (weak), 1709 (very strong), 1678 (very frequencies (cm⁻¹): 3534 (medium), 2924 (strong), 2398 Observable absorption maxima occur at the following in chloroform is shown in the accompanying drawing. The infrared absorption spectrum of tylactone of C23H38O5 and a molecular weight of about 394. gen, 9.78; oxygen, 20.3%. It has an empirical formula

spray, either concentrated or dilute (50%), may be used

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The $R_{ extsf{I}}$ of tylactone in this system is about tography in a benzene:ethyl acetate (4:1) solvent R value of about 0.59 on silica-gel thin-layer chromato give 3,5-di-0-acetyltylactone. This compound has an at 60° for 1/2 hour and then concentrated under vacuum 52 (5 ml) was added to the residue; the solution heated then concentrated to dryness under vacuum. Methanol allowed to stand at room temperature for 16 hours and anhydride (4 ml) was added. The resulting mixture was Example 2, was dissolved in pyridine (4 ml). Acetic 20 Tylactone (200 mg), prepared as described in

3,5-Di-O-Acetyltylactone

Example 3

B = perzene: ethyl acetate (3:2)DSolvent: A = benzene: ethyl acetate (4:1)

		Silica gel	:muibəM ⁵
	0.0	-	Tylosin
0.0	_		Tylactone
29.0	05.0		Сомроила
Ē	d <u>A</u>		Биноотор

RE Value

Thin-Layer Chromatography of Tylactone

Table 1

Table 1. approximate Rf values of tylactone are summarized in the chromatography, UV detection is convenient. del plates with a fluorescent background are used in appears initially as a yellow-to-brown spot. If silicafor detection. With this detection system tylactone

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Examples 4-7

3,5-Di-O-propionyltylactone, prepared according anhydride.

3,5-Di-O-isovaleryltylactone, prepared according isovaleric anhydride.

3,5-Di-O-benzoyltylactone, prepared isovaleric anhydride.

3,5-Di-O-benzoyltylactone, prepared according isovaleric anhydride.

to the procedure of Example 3, but using benzoic anhydride.

3,5-Di-O-(n-butyryl)tylactone, prepared according to the procedure of Example 3, but using

according to the procedure of Example 37 and n-

Example 8

Preparation of Tylosin from Tylactone

produced tylosin but which was blocked in macrolide

ting closure was fermented according to the procedure

described in Example 1, Section A, except that a temfermentation 48 hours after inoculation. The ferment

tylosin was produced, i.e. about three additional days.

The presence of tylosin is determined by testing

samples of the broth against organisms known to be

sensitive to tylosin. One useful assay organism is

Staphylococcus aureus ATCC 9144. Bioassay is consensitive to tylosin, An automated turbidometric

sensitive to tylosin. One useful assay organism is Staphylococcus aureus ATCC 9144. Bioassay is conveniently performed by an automated turbidometric method, by thin-layer chromatography or by high-performance liquid chromatography with UV detection.

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CLAIMS

Tylactone or an ester derivative thereof carbon, nitrogen and inorganic salts. SI myces fradiae NRRL 12188 and assimilable sources of A culture medium which comprises Strepto-Streptomyces fradiae NRRL 12188. comprises cultivating Streptomyces fradiae NRRL 12188. OΤ A process according to claim 1 which by esterification. conditions to produce tylactone, followed, optionally, and inorganic salts under submerged aerobic fermentation medium containing assimilable sources of carbon, nitrogen, producing mutant or recombinant thereof, in a culture Streptomyces fradiae NRRL 12188, or a tylactonean ester derivative thereof, which comprises cultivating 1. A process for preparing tylactone, or

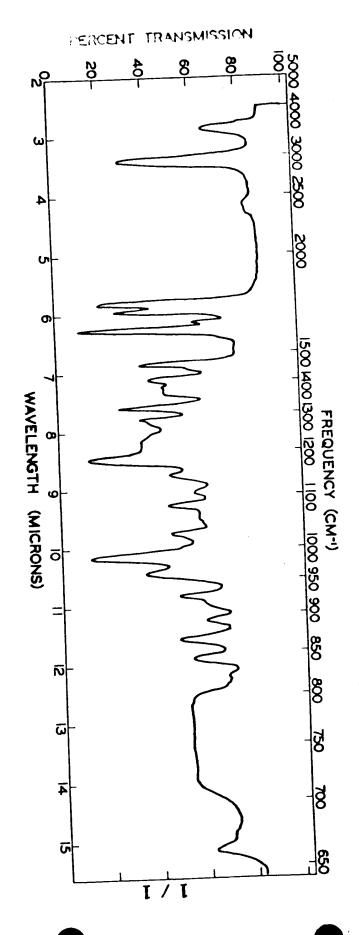
whenever prepared by a process according to either of claims

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EUROPEAN SEARCH REPORT



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temity.	The present search report has been drawn up for all claims	<u> </u>
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CATEGORY OF CITED DOCUMENTS X: particularly relevant P: Internediate document T: theory or principle underlying P: Internediate document T: theory or principle underlying T: the underlying T: the underlying T: the und		
C 15 P 17/08 C 07 D 313/06 A 61 K 31/365		
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gida egili. Sessa kuloji. Sessa kuloji.	* Complete article *	
(c is p 17/08;	1963-1965, edit, by Pharmaceutical Society of Japan Society of Japan Characterization of a new 16-membered lactone, protylonolide, from a mutant of tylosin-producing strain, streptomyces fradiae KA-atrain, streptomyces fra	·
(C IS b 11/08/) C O1 D 313/00 V 01 K 31/302	CHEMICAL & PHARMACEUTICAL BULLETIN 1,2,5 vol. 28, no. 6, June 1980, pages	X
376/16 // /	Citation of document with indication, where appropriate; or casim	Calegory
CLASSIFICATION OF THE APPLICATION on CLA	DOCUMENTS CONSIDERED TO BE RELEVANT	

Office européen des brevets

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EUROPEAN PATENT SPECIFICATION

CO1D 313/00' (3) luf. Cl.3: A 61 K 31/365, A8.E0.Af :noisonication of patent specification: 14.03.84

C1581/24) C15P 17/08 //(C12P17/08,

(I) Application number: 81302964.2

18.80.05 : gnilft to sted (5)

- .ebilorsem e gnineparing a macrolide.
- (2U) 28234 ensibnl zilogensibnl 307, East McCarty Street (13) Proprietor: ELI LILLY AND COMPANY
- Inventor: Seno, Eugene Thomas (SU) 09234 ensibnl siloqensibnl 7446, Sunset Lane (1) Inventor: Baltz, Richard Henry
- Colney Lane Morwich NR4 7UH (GB) Cottage No. 1 John Innes Institute
- Windlesham Surrey GU20, 6PH (GB) Etl Wood Manor (4) Representative: Hudson, Christopher Mark et al.

- TT6281 8U 08.T0.S0 :Yfinoin9 (8)
- r\sq nitellu8 s8. r0. a0 (3) Date of publication of application:
- 14,03.84 Bulletin 84,111 Publication of the grant of the patent:
- BE CH DE FR GB IT LI LU NL SE (bg) Designated Contracting States:

producing strain, streptomyces fradiae KA-427 protylonolide, from a mutant of tylosincharacterization of a new 16-membered lactone. bns noitslos!" : ARUMO IH2OTA2 edit. by Pharmaceutical Society of Japan, ,8361-5361 pages 1980, pages 1963-1965, CHEMICAL & PHARMACEUTICAL BULLETIN, :befic secrences cited:

paid. (Art. 99(1) European patent convention). be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall Note: Within nine months from the publication of the mention of the grant of the European patent, any person may

Courier Press, Learnington Spa, England.

pound with a stoichiometric quantity (or a slight such as, for example, treatment of the comfication techniques generally known in the art The derivatives can be prepared by caster the structure 7: tone for convenience hereinafter. Tylactone has separated and purified by known techniques dideoxytylonolide, which will be called tylac-Once formed, the acyl derivatives can by preparation of the macrolide 20-dihydro-20,23described by Okamoto et al. in U.S. 4,092,473 This invention relates to a process for the

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derivatives which have structure 2: It is useful in the preparation of related acyl

wherein R and $R_1 = an$ acyl moiety. 5

try of the compounds is identical to that of the structures given herein, the stereochemisno stereochemical assignments are indicated in macrolide antibiotics can be prepared. Although useful intermediates from which 16-membered The compounds of structures 1 and 2 are

tylactone are useful as intermediates in the preknown in the art. The acyl ester derivatives of treatment with acylating agents using methods hydroxyl groups to give acyl ester derivatives by Tylactone can be esterified at the 3- and 5-UISOIA

enzymatically using procedures such as those carbodiimide. Acylations can also be carried out hydrating agent such as N.N'-dicyclohexylusing a mixture of an organic acid and a deorganic acids. Acylation can also be achieved by other acid scavenger) and active esters of halides (usually in combination with a base or Typical acylating agents include anhydrides, paration of new macrolide antibiotics.

eniuper furoing and the growth requiremi mulbem ent to estructivents of the medium in Such trace elements commonly occur as should also be included in the culture medium; growth and development of the organism Essential trace elements necessary for the carbonate, sulfate and nitrate ions.

magnesium, calcium, ammonium, chloride;

capable of yielding iron, potassium, sodium, culture media are the customary soluble saits

inorganic salts which can be incorporated in the

meal and amino acids. Among the nutrient

sources include corn meal, soybean meal, fish

and oils such as soybean oil. Preferred nitrogen such as dextrin, glucose, starch, and com meal

large-scale fermentation include carbohydrates

Thus, for example, preferred carbon sources in

however, certain culture media are preferred.

optimal yield, and ease of product isolation,

number of media. For economy in production,

Streptomyces tradiae can be any one of a

si bruodmoo beniesb and to muome leitness

ditions in a suitable culture medium until a sub-

this compound under submerged serobic constrain of Streptomyces fradiae which produces Tylactone can be prepared by culturing a maleic, fumaric, malonic and phthalic acids. derived from dicarboxylic acids such as succinic, moiety. Suitable esters also include hemi-esters halogen, nitro and lower alkoxy on the aromatic acids optionally bearing substituents such as stalkyl-sultonic acids, the aryl- and stalkyland 2-thienylacetic acids, and alkyl-, aryl-, and zoic, phenylacetic, phenoxyacetic, mandelic hexylpropionic, 1 - adamantanecarboxylic, bencarboxylic, cyclohexanecarboxylic, β - cyclocuronic, alkoxycarbonic, stearic, cyclopropanechloroacetic, propionic, butyric, isovaleric, giuderived from acids such as formic, acetic

Representative suitable esters include those

and of inorganic acids, such as sulfuric and carbonic acids of from 1 to 18 carbon atoms. heterocyclic carboxylic, sulfonic and alkoxyincluding aliphatic, cycloaliphatic, aryl, aralkyl. Useful esters are those of organic acids

standard procedures such as extraction be isolated from the reaction mixture by substantially complete. The ester derivative can ture for from 1 to 24 hours until esterification is

pyridine) at about 0°C to about room temperaanhydride, in an organic solvent (for example, excess) of an acylating agent, such as an acyl

chromatography and crystallization.

produced.

phosphoric acids.

The culture medium used to grow the

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graphic Science, 16, 492-495 (1978)]. [see, for example, J. H. Kennedy in J. Chromatochromatography with a UV detection system

qiosbs yd berinuq ed ysm i ,benisido si lio ns phase under vacuum to give crystals or an oil, it petroleum ether, concentrating the organic with a suitable solvent such as amyl acetable of the broth (generally without pH adjustment) cation of the filtered broth involves extracting processes. A preferred technique for purifitechniques may be used in the extraction filtered broth and the mycelial cake. A variety of be recovered from the fermentation medium by aerobic fermentation conditions, tylactone can Following its production under submerged

fermentation broth and extraction of both the of the fermentation broth or S intration of the therefore, can be accomplished by 1) extraction which it is produced. Recovery of tylactone; ni mulbem ant ni aldulor radionation yem of the limited solubility of tylactone in water, it. methods used in the fermentation art. Because

capable of producing tylosin except that it is strain which either produces tylosin itself or is microorganism can be a Streptomyces fradiae converting microorganism The bioconverting tylosin by adding it to a growing culture of a bioexample, tylactone (1) can be bioconverted to macrolide antibiotics can be prepared. For usetul intermediates from which 16-membered The compounds of structures I and 2 are tion chromatography.

strain a mutagen and screening survivors can be obtained by treating a tylosin-producing except that it is blocked in tylactone formation A strain which is capable of producing tylosin blocked in tylactone formation.

vivors to determine if they produce tylosin. to small shake-flask cultures of the selected sur-These strains are identified by adding tylactone strains are also unable to produce tylactone. tylosin are further screened to determine which Those survivors which are unable to produce for those which are unable to produce tylosin.

.anibinaugosonin -onlin-'N-lydram-N si aniens betoelee edt A typical mutagen which may be used to obtain strains which are capable of producing tylosin. NBBL 2703 are examples of Streptomyces Streptomyces fradiae strains NRRL 2702 and

the metabolic pathway of tylosin can be tylactone portion or the added sugar moieties, for metabolic studies. By labeling either the useful in the preparation of labeled compounds The compound of structure 1 is especially

of this invention, the following examples are In order to illustrate more fully the operation ascertained.

Example 1 :bebivorq

3.0) notiulos sint to notition A statistical description (3.0) NRRL 12188 was dispersed in 1-2 ml of A lyophilized pellet of Streptomyces fradiae A. Shake-flask Fermentation of Tylactone

the broth, using high-performance liquid to saldmes gnitsat yd noitstnamples of Production of tylactone can be followed

28°C and one atmosphere of pressure). production should be about 30% or above (at production the percent of air saturation for tank the culture medium. For efficient antibiotic

culture processes, sterile air is bubbled through 28°C.

temperatures between about 10° and about

teristic of tylactone production are a part of this

fradiae NRRL 12188 which retain the charac-

mutants and recombinants of Streptomyces guanidine. All natural and induced variants,

gamma rays, and N-methyl-N'-nitro-N-nitroso-

mutagens, such as ultraviolet light, X-rays,

with various known physical and chemical

12188 strain may be obtained by treatment recombinants, mutants or variants of the NRRL

12188 are subject to variation. For example,

characteristics of Streptomyces fradise NRRL

from which it is available to the public under the

North University Street, Peoria, Illinois, 61604, tural Research, North Central Region, 1815

Northern Regional Research Center, Agricul-

part of the stock culture collection of the

microorganism has been deposited and made

strain of Streptomyces fradiae. A culture of this

amounts of tylosin, but produces tylactone as a

The new microorganism produces only minimal myces fradiae strain which produces tylosin.

obtained by chemical mutagenesis of a Strepto-

culturing a new microorganism which was

that used for larger fermentations, but other

for the vegetative inoculum can be the same as

transferred to a larger tank. The medium used

organism. The vegetative inoculum is then

a fresh, actively growing culture of the

or mycelial fragments of the organism to obtain

volume of culture medium with the spore form

lisms a gnistinoculating a small

to use a vegetative inoculum. The vegetative

the spore form of the organism, it is preferable

associated with inoculation of large tanks with

Because of the time lag in production commonly

may be obtained by shake-flask culture.

tanks is preferred. Small quantities of tylactone

tylactone submerged aerobic fermentation in

2000) to large-scale fermentation media if

agent such as polypropylene glycol (M.W. about

meofirms no to (J\im 2.0 .e.i) strutome lisms bbe

ments of the organism. It may be necessary to

For production of substantial quantities of

The method of this invention comprises

The new microorganism is also classified as a

accession number NRRL 12188.

As is the case with other organisms, the

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major component.

media can also be used.

foaming becomes a problem.

S. fradiae NRRL 12188 can be grown at

As is customary in aerobic submerged appears to occur at temperatures of about 40°C. Optimum production of tylactone

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	Nater	
: 810.0	Lecithin (crude)	
3. 0	Soybean oil (crude)	
£.0	CaCO,	
9.0	Yeast extract	
3 .0	Soybean meal	
0.1	Corn steep liquor	
(%) InvomA	Ingredient	

The pH was adjusted to 8.5 with 50% NaOH plution.

This second-stage vegetative medium was incubated in a 68-liter tank for about 47 hours at 29°C.

Incubated second-stage medium (4 L) thus prepared was used to inoculate 40 liters of sterile production medium having the following composition:

06.06	Water
60.0	Lecithin
3.15	Soybean oil (crude)
2.10	Seet molasses
4 0.0	(NH ⁴) ³ HbO ⁴
01.0	NaCi
15.0	CaCO ₃
26.0	Corn gluten
73.f	Corn meal
26.0	Fish meal
(%) InuomA	Ingredient

The PH was adjusted to 7.5 with 50% NaOH solution.

The inoculated production medium was allowed to ferment in a 68-liter tank for about 5 allowed to ferment in a 68-liter tank for about 5 days at a temperature of 28°C. The dermentation medium was aerated with sterile air to keep the dissolved oxygen level between about 30% and 50% and about 30% rounsentional agitators at about 300 rpm.

muse used to inoculate a vegetative medium:
(350 ml) having the following composition:

Ingredient

Ingredient

avitatanay s Mayitsdaatl		_
Deionized water	32.79	
Soybean oil (crude)	94.0	
coco,	€.0	
Soybean grits	3 .0	
Yeast extract	3 .0	
Corn steep liquor	0.1	
Ingredient	(%) InvomA	

Alternatively, a vegetative culture of 5 fradiae NRRL 12188 preserved, in 1-ml volumes, in liquid nitrogen was rapidly thawed and used to inoculate the vegetative medium. The inoculated vegetative medium was incubated in a 500-ml Erlenmeyer flask at 29°C. for bated in a 500-ml Erlenmeyer flask at 29°C, for 300 pm.

This incubated vegetative medium (0.5 ml) was used to inoculate Γ ml of a production was used to inoculate Γ medium having the following composition:

· · · · · · · · · · · · · · · · · · ·	
Deionized water	98.16
Soybean oil (crude)	0.5
c _a cO _s	2.0
, MH ₄)2HPO4	4 0.0
NaCI	r.o
Corn gluten	6.0
lsəm dzi3	6.0
Com meal	3 .1
sessiom teed	2.0
Ingredient	(%) InnomA

The inoculated fermentation medium was incubated in a 50-ml bottle at 29°C, for about 6 days on a closed-box shaker at 300 rpm.

B. Tank Fermentation of Tylactone of In order to provide a larger volume of inoculum, 60 ml of incubated vegetative medium, prepared in a manner similar to that described in section A, was used to inoculate 38 L of a second-stage vegetative growth medium having the following composition:

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ΟΕ

Tylactone is nearly insoluble in water, but is titratable groups.

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rescent background are used in the chighter tography, UV detection is convenient. The approximate Rt values of tylacions are summarized in Table 1 system tylactone appears initially as a yellow to-brown spot. If silica-gel plates with a flucby silica-gel thin-layer chromatography, Suffuggacid spray, either concentrated of dilute [505] may be used for detection. With this detection Tylactone can be distinguished from tylosin and dimethyl sulfoxide. form, diethyl ether, petroleum ether, benzene

methanol, ethanol, dimethylformamide, chloro

soluble in organic solvents such as acetone,

Thin-Layer Chromatography of Tylactone I 3J8AT A Commence of the Commence of Service Company

0.0 nisolyT 0.0 Tylactone 29.0 05.0 DunodmoD ٩٧

Af Value

B=benzene:ethyl acetate (3:2) bSolvent: A=benzene:ethyl acetate (4:1) ^eMedium: Silica gel

Example 3

.E.O tuods si mətaya solvent system. The Rt of tylactone in this tography in a benzene:ethyl acetate (4:1) of about 0.59 on silica-gel thin-layer chromaacetyltylactone. This compound has an R, value centrated under vacuum to give 3,5-di-Otion heated at 60° for ½ hour and then con-(5 ml) was added to the residue; the solutrated to dryness under vacuum. Methanol temperature for 16 hours and then concennoor te bnets of bewolle sew enutxim gnitluser Acetic anhydride (4 ml) was added. The Example 2, was dissolved in pyridine (4 ml). Tylactone (200 mg), prepared as described in 3,5-Di-O-Acetyltylactone

using propionic anhydride. according to the procedure of Example 3, but 3,5 - Di - O - propionyltylactone, prepared Examples 4---7

according to the procedure of Example 3, but 3,5 - Di - O - isovaleryltylactone, prepared

3,5 - Di - O - benzoyltylactone, prepared using isovaleric anhydride.

using benzoic anhydride. according to the procedure of Example 3, but

using n-butyric anhydride. according to the procedure of Example 3, but 3,5 - Di - O - (n - butyryl)tylactone, prepared

Example 2

Isolation of Tylactone

give about 2 g, m.p. 162-163°C. tallized from benzene-hexane or hot hexane to containing tylactone were combined and evaporated under vacuum. Tylactone was crysto separate and isolate tylactone. Fractions stances, then with benzene:ethyl acetate (9:1) eluted with benzene to remove lipid subacid spray for detection. The column was first acetate (3:2) solvent system and conc. sulfuric layer chromatography, using a benzene:ethyl benzene. Elution is monitored by silica-gel thin-Davison Chemical Co.) column, packed with 5.25 x 36 in. silica-gel (Grace, grade 62, benzene solution was chromatographed over a The oil was dissolved in benzene (5 L). The was concentrated under vacuum to give an oil. reading at 282 nm but no antimicrobial activity) acetate extract (which has a high optical density extracted with amyl acetate (400 L). The amyl of 2% sodium hydroxide. The filtrate was filtrate was adjusted to about 9 the addition earth, Johns Manville Corp.). The pH of the filter aid (3% Hyflo Supercel, a diatomaceous described in Example 1, was filtered using a Fermentation broth (1600 L), obtained as

panying drawing. tone in chloroform is presented in the accom-The infrared absorption spectrum of tylac-

The infrared absorption spectrum of ty-C₂₃H₃₈O₅ and a molecular weight of about 394. oxygen, 20.3%. It has an empirical formula of :%7.9 ,nodrogen, 70%; hydrogen, 9.7%; following approximate percentage elemental melts at about 162-163°C. It has the from hexane or ethyl acetate-hexane and which Tylactone is a white solid which crystallizes

1025 (medium), 984 (very strong), 896 (Ilsms view), 1049 (wedium), 1049 (wery small), 1379 (small), 1316 (strong), 1284 (medium), 1979 (serong), 1143 (strong), 1103 1458 (strong), 1441 (shoulder), 1404 (strong), (very strong), 1626 (small), 1592 (very strong), (Megk), 2353 (Wedk), 1709 (very strong), 1678 maxima occur at the following frequencies (cm⁻¹): 3534 (medium), 2924 (strong), 2398 lactone in chloroform is shown in the notoclon original observable absorption

(lleme) 188 bne (lleme viev) (strong), 923 (medium), 911 (shoulder), 859 (strong), 868 (medium), 840 (medium), 820

.(63% = 560). mn 282 tuode te mumixem noitqrozde tylactone in neutral ethanol exhibits an The ultraviolet absorption (VU) spectrum of

Tylactone has the following specific rotation:

 $[\alpha]_{2e}^{\Sigma e} - 22.23^{\circ} (c 1, CH_3OH).$

aqueous dimethylformamide indicates it has no Electrometric titration of tylactone in 66%

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Revendications

1. Procédé de préparation de tylactone ou d'un de ses dérivés esters, caractérisé en ca qu'il consiste à cultiver la souche Streptomyces fradise NRRL 12188, un de ses mutants ou recombinants producteurs de tylactone, dans un milieu de culture contenant des sources assimilables de carbone, d'asote et de sels inorbables de carbone, d'asote et de sels inorbables des culture conditions de fermentation aérobie submergée pour produire de la tylactone, cette culture étant éventuellement suivie d'une estérification.

2. Procédé suivant la revendication 1, caractérisé en ce qu'il consiste à cultiver la souche Streptomyces fradiae NRRL 12188.

Patentansprüche

1. Verfahren zur Herstellung von Tylacton oder einem Esterderivat hiervon, dadurch gekennzeichnet, daß man Streptomyces fradise nach Streptomyces fradise AMRL 12188 oder eine tylactonbildende Mutante oder Rekombinante hiervon in einem Kulturmedium, das assimilierbare Quellen für Kohlenstoff, Stickstoff und anorganische Salze enthält, unter submersen aeroben Fermentationscheft, unter submersen aeroben Tylacton züchtet und gegebenenfalls dann eine Veresterung vornimmt.

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß man Streptomyces fradiae NRRL 12188 züchtet.

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conveniently performed by an automated Staphylococcus aureus ATCC 9144. Bioassay is to tylosin. One useful assay organism is broth against organisms known to be sensitive tylosin is determined by testing samples of the about three additional days. The presence of stantial amount of tylosin was produced, i.e. fermentation was then continued until a subthe fermentation 48 hours after inoculation. The ture of 28°C was used. Tylactone was added to Example 1, Section A, except that a temperaaccording to the procedure described in in macrolide ring closure was fermented merly produced tylosin but which was blocked A Streptomyces fradiae strain which for-Preparation of Tylosin from Tylactone

egraphy or by high-performance liquid chromatography with UV detection.

urbidometric method, by thin-layer chroma-

Claims

1. A process for preparing tylactone, or an ester derivative thereof, which comprises cultivating Streptomyces tradise NRRL 12188, or a tylactone-producing mutant or recombinant thereof, in a culture medium containing assimilable sources of carbon, nitrogen, and incoganic salts under submerged serobic fermentation conditions to produce tylactone, followed, optionally, by esterification.

2. A process according to claim 1 which comprises cultivating Streptomyces fradiae NRRL 12188.

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